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In vitro insertional mutagenesis with a selectable DNA fragment

(Recombinant DNA; mutagenesis with DNA linkers; antibiotic resistance; genetic mapping; Ω fragment; interposon; translational/transcriptional termination)

Pierre Prentki* and Henry M. Krisch**

Département de Biologie Moléculaire, Université de Genève, 30 quai Ernest-Ansermet, 1211 Geneva 4 (Switzerland) Tel. 022/219355: ext. 2126

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SUMMARY

A new method for in vitro insertional mutagenesis of genes cloned in *Escherichia coli* is presented. This simple procedure combines the advantages of in vitro DNA linker mutagenesis with those of in vivo transposition mutagenesis. It makes use of the Ω fragment, a 2.0-kb DNA segment consisting of an antibiotic resistance gene (the Sm^r/Spc^r gene of the R100.1 plasmid) flanked by short inverted repeats carrying transcription and translation termination signals and synthetic polylinkers. The Ω fragment is inserted into a linearized plasmid by in vitro ligation, and the recombinant DNA molecules are selected by their resistance to streptomycin and spectinomycin. The Ω fragment terminates RNA and protein synthesis prematurely, thus allowing the definition and mapping of both transcription and translation units. Because of the symmetrical structure of Ω, the same effect is obtained with insertions in either orientation. The antibiotic resistance gene can be subsequently excised from the mutated molecules, leaving behind its flanking restriction site(s).

INTRODUCTION

Several methods have been developed to isolate mutants of recombinant plasmids in *E. coli*. They usually involve inactivation of the cloned genes by insertion of foreign DNA sequences: transposable elements in vivo, or synthetic DNA linkers in vitro (Heffron et al., 1978). In a previous communication (Prentki and Krisch, 1982) we described the construction of a plasmid, called pKP6, that contained an antibiotic-resistance gene flanked by inverted repeats of a DNA linker sequence, and suggested that this fragment could be used for in vitro mutagenesis of DNA, with the advantage of a direct

* Present address: Department of Molecular Biology, University of Southern California, Los Angeles, CA 90089-1481 (U.S.A.) Tel. 213-743-8431.
** To whom correspondence and reprint requests should be addressed.

Abbreviations: Ap, ampicillin; bp, base pairs; Cm, chloramphenicol; DTT, dithiothreitol; EtBr, ethidium bromide; kb, kilobase pairs; LA, Luria broth agar; PAGE, polyacrylamide gel electrophoresis; ^{r} (superscript), resistance; SDS, sodium dodecyl sulfate; Sm, streptomycin; Spc, spectinomycin; SSC, 0.15 M NaCl, 0.015 M Na_3 · citrate pH 7.6; Tc, tetracycline; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactoside; [ ], indicates plasmid-carrier state.

selection of the altered molecules. An analogous construction has been made independently by Vieira and Messing (1982).

Ideally, a DNA fragment to be used for insertional mutagenesis should have three properties: (*i*) be selectable; (*ii*) abolish the expression of the genetic unit into which it has been inserted; and (*iii*) be easy to localize. We report here the construction and the use of a plasmid, called pHP45Ω, which carries a DNA fragment ("Ω") incorporating these three properties.

MATERIALS AND METHODS

(a) Bacterial strains and plasmids

E. coli C600 $r_K^- m_K^-$ *su*II and HB101 were the standard strains used for bacterial transformations by plasmid DNA. The chromosomal *lac* deletion strain MC1061 (*araD*139, *Δara-leu*7697, *ΔlacX*74, *galU*$^-$, *galK*$^-$, *hsr*$^-$, *hsm*$^+$, *strA*) was provided by M. Casadaban. The plasmids pHP34 and pKP6 have been described previously (Prentki and Krisch, 1982). The plasmid pKTH604 was obtained from R.F. Pettersson. The construction of pHP45 is outlined in the legend to Fig. 1.

(b) Preparation and in vitro manipulation of plasmid DNA

Most of the techniques used in this report have been previously described by Prentki and Krisch (1982). Linearization of plasmid DNA using a restriction enzyme with many recognition sites in the molecule was performed in the presence of EtBr (Parker et al., 1977). Typically, 5–10 μg of plasmid DNA was incubated with 20–40 units of restriction enzyme at 37°C for 5 min in the appropriate buffer supplemented with EtBr to 90 μg/ml. The reaction was terminated by phenol/chloroform extraction, and EtBr was removed by isoamyl alcohol extraction followed by ethanol precipitation of the DNA. Since the sensitivity of different restriction enzymes to inhibitory effects of EtBr was found to vary, it was necessary to determine the optimal conditions for linearization for each one.

DNA fragments to be purified were separated by PAGE, visualized by staining with methylene blue (0.10%), and recovered from excised bands by electrophoresis into a dialysis bag (McDonell et al., 1977). Filling-in of staggered restriction sites was performed as described by Maniatis et al. (1982).

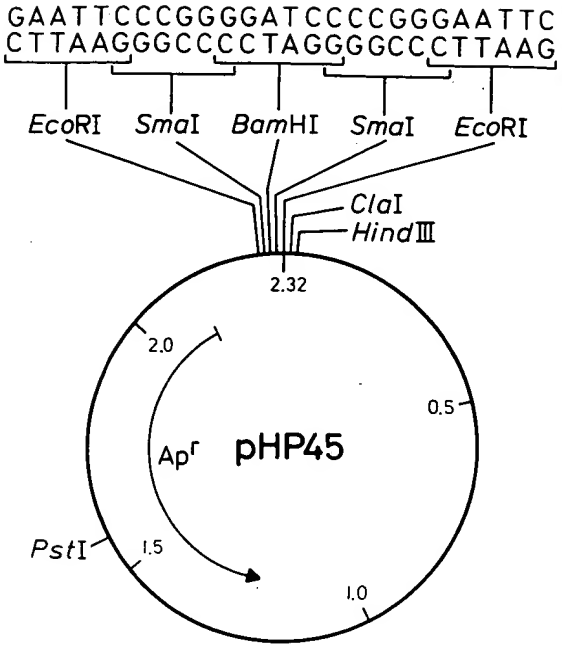


Fig. 1. Structure of the plasmid pHP45. This plasmid was constructed by modifying pBR322 in two ways: (1) by the insertion at the *Eco*RI site of a 20-bp palindromic sequence consisting of a *Bam*HI site flanked by *Sma*I and *Eco*RI sites; (2) by in vitro recircularization between the *Pvu*II and the filled-in *Hin*dIII sites, thus deleting a 2.03-kb segment including the Tc^r gene and the second *Bam*HI site. pHP45 was chosen among several Ap^{r}Tc^s transformants because its *Hin*dIII site had been reconstituted during the ligation, presumably because of a 1-bp deletion at the junction between the *Pvu*II site and the filled-in *Hin*dIII site. The unique *Hin*dIII site of pHP45 makes it appropriate for our simplified insert sequencing strategy (Prentki and Krisch, 1982). DNA fragments with 5′-GATC protruding ends can be cloned into the unique *Bam*HI site of pHP45, and be recovered upon digestion with *Sma*I (*Xma*I) or *Eco*RI.

(c) Transformation

Competent *E. coli* cells were prepared and transformed according to Norgard et al. (1978). Selection for resistance to antibiotics was performed on LA plates supplemented with the appropriate antibiotic (Ap, 25 μg/ml; Tc, 25 μg/ml; Sm, 20 μg/ml; Spc, either 20 μg/ml or 100 μg/ml). Double selection for Sm^r and Spc^r greatly reduces the level of spontane-

ously resistant colonies observed with either antibiotic separately. Selection for Spc^r can be applied in strains carrying chromosomal Str^r. The ability to utilize lactose was determined on MacConkey indicator plates, or on M9 plates supplemented with X-gal at 40 μg/ml.

(d) In vivo protein synthesis

Cellular protein synthesis was analyzed as described previously (Krisch and Selzer, 1981) by SDS-PAGE (Laemmli, 1970).

(e) Pulse-labeling and extraction of RNA

Bacteria were grown in M9 medium (Adams, 1959) supplemented with glucose (0.4%), casamino acids (10 mg/ml), thiamine (0.5 μg/ml) and thymine (20 μg/ml). At an A_{450} of 0.3, aliquots of the culture (2.5 ml) were exposed to [5-^{3}H]uridine (New England Nuclear: spec. act. 25 Ci/mmol; 66 μCi/ml) for 1 min. The pulse was terminated by the addition of an equal volume of a NaN_3 (20 mM) and uridine (1 mg/ml) solution and rapid cooling in dry ice/ethanol. Total RNA was prepared as described by Dennis and Nomura (1975).

(f) RNA-DNA hybridizations

The same molar amount (167 fmol) of each probe fragment was loaded onto nitrocellulose filters. Duplicate filters carrying no DNA or DNA of the probes were pre-hybridized with Denhardt (1966) solution: bovine serum albumin, polyvinyl pyrolidone, and Ficoll (all at 0.02%), supplemented with SDS (0.2%) and yeast tRNA (100 μg/ml), and incubated for 4 h at 67.5°C. The solution was then replaced with 10 μg [^{3}H]RNA in 2 ml 2 × SSC buffer supplemented with SDS (0.1%) and yeast tRNA (100 μg/ml). The hybridization vials were incubated at 67.5°C for 18 h with gentle agitation. The filters were washed three times in 2 × SSC and incubated for 1 h at 25°C in 2 × SSC containing RNase A (20 μg/ml) and RNase T1 (25 units/ml). After three more washings in 2 × SSC, the filters were dried and counted in a liquid scintillation counter.

RESULTS

(a) Construction of pHP45Ω

The structure of pHP45Ω is shown in Fig. 2a. The series of in vitro reactions by which pHP45Ω was assembled is presented in detail elsewhere (Prentki, 1983). Four DNA fragments were involved in the construction of pHP45Ω:

(1) The Sm^r/Spc^r gene

This antibiotic resistance gene (*aadA*$^+$) was carried on a 1.7-kb *Pvu*II-*Hin*dIII fragment from the R100.1 plasmid.

(2) The transcription-termination sequences

These sequences were obtained from the plasmid pMJK4-18 (Krisch and Selzer, 1981), which contains the carboxyl-terminal portion of bacteriophage T4 gene 32. A 118-bp segment of the T4 insert was produced by the restriction enzymes *Bst*NI and *Hin*dIII, and a *Bam*HI synthetic linker sequence was added to the filled-in *Bst*NI site (B. Allet and H. Krisch, unpublished). The *Bam*HI site was in turn filled-in to fuse the transcription terminators to the Sm^r/Spc^r fragment.

The sequence of this transcription-termination fragment has been established (Krisch and Allet, 1982; Fig. 2b). It carries the last eight codons of gene 32, two consecutive translation termination codons, and a characteristic transcription termination sequence. That this sequence is indeed active as a termination site for RNA polymerase was shown both in vivo (Prentki, 1983) and in vitro (Krisch, H. and Selzer, G., unpublished).

(3) The translation-termination sequences

Terminator codon fragment was obtained from the plasmid pKTH604 (Pettersson et al., 1983) on a 21-bp *Bam*HI-*Hin*dIII synthetic DNA fragment with nonsense (TGA) codons in all three phases.

(4) The vector

The replicon used in the construction of pHP45Ω is pHP45 (Fig. 1).

(b) Verification of the structure of pHP45Ω

The presence of two terminator sequences in inverted orientation flanking the antibiotic resistance

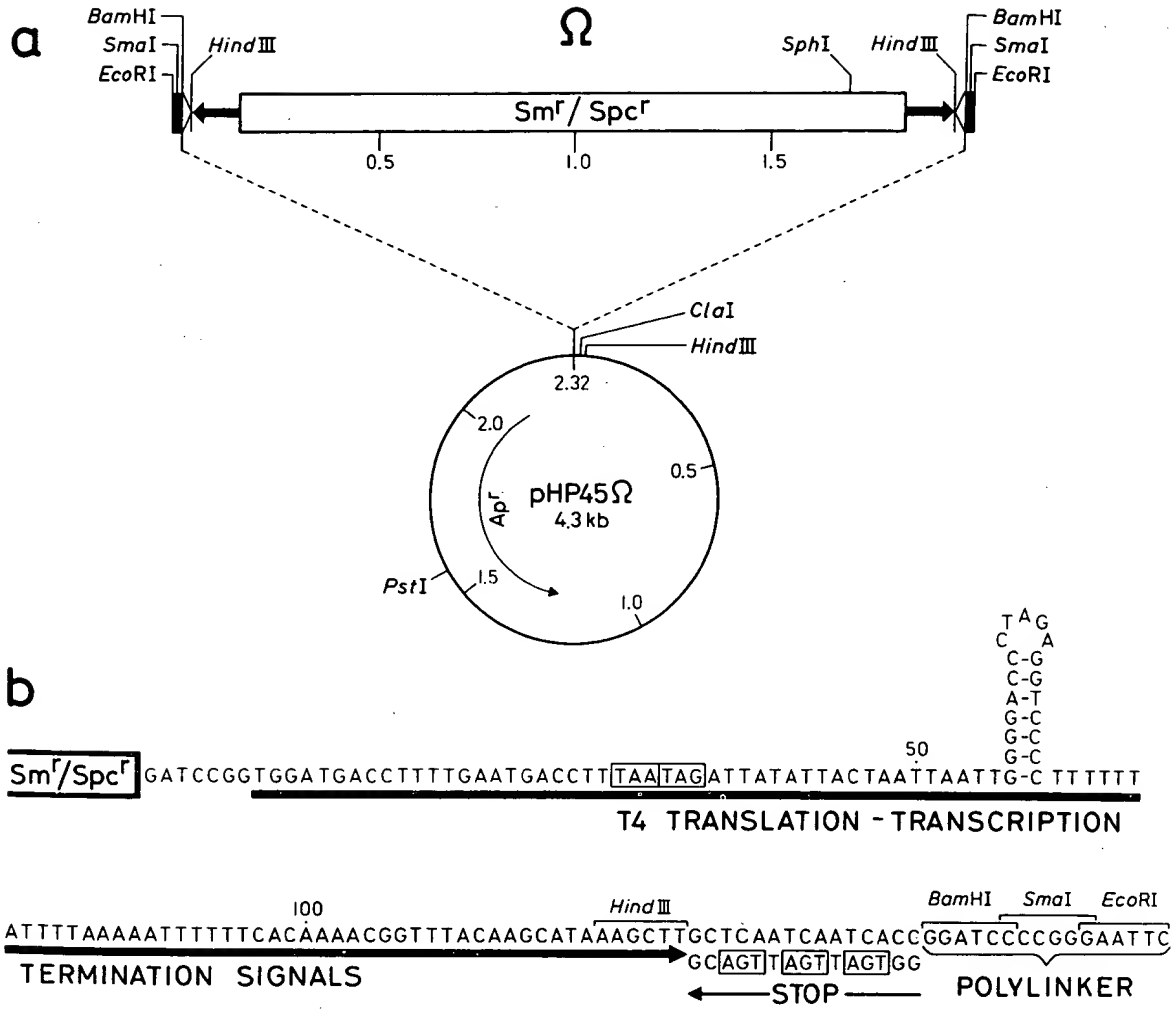


Fig. 2. Structure of the Ω element. (a) Recombinant plasmid pHP45Ω. The pHP45 vector (Fig. 1) is represented as a circle and the Ω fragment in linear form. The Sm^r/Spc^r segment from R100.1 (open rectangle) is flanked by short inverted repeats carrying the T4 transcription-termination signals (arrows), the translational stop signals (triangles), and the polylinker (black boxes). (b) Nucleotide sequence (5′ → 3′) of the inverted repeat of Ω. The T4 segment (heavy arrow) contains the last seven codons of gene 32 and the tandem translational stop codons TAA and TAG (boxed). The transcription-termination sequence (hairpin) is oriented so as to block transcription termination coming out of the Ω fragment. It is followed by the *Hin*dIII-*Bam*HI synthetic DNA fragment from pKTH604, with TGA nonsense codons in all three phases on the opposite strand, and by the polylinker.

gene was demonstrated by electron microscopy. Plasmid pHP45Ω DNA was linearized with *Pst*I, denatured, and then examined for the presence of snap-back structures. Fig. 3a shows two such structures. The size of the loop (1.71 kb), the length of the double-stranded stem (0.16 kb), and the length of the arms flanking the stemloop structure (0.71 and 1.49 kb) are in agreement with those expected from Fig. 2. The same stemloop structure was observed with circular single-stranded pHP45Ω DNA, but the arms were replaced with a second loop of a size identical to that of the pHP45 plasmid vector (Fig. 3b).

A restriction analysis of plasmid pHP45Ω DNA is shown in Fig. 4. As expected, digestions with *Eco*RI (Fig. 4a), *Sma*I (b) and *Bam*HI (c) result in almost identical patterns of two bands: the largest corresponds in size to pHP45, and the other to the 2.0-kb Sm^r/Spc^r Ω fragment. Digestion of pHP45Ω with *Hin*dIII (d) replaces the 2.0-kb fragment by a band of approximately 1.95 kb. This reduction in size corresponds to the removal by *Hin*dIII of the 21-bp

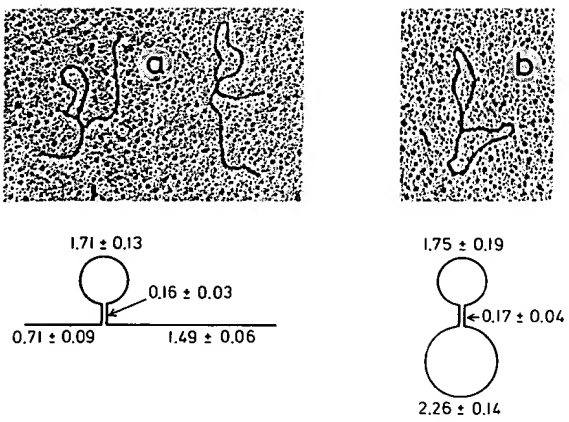


Fig. 3. Electron micrographs of snap-back structures observed in single-stranded DNA of pHP45Ω treated (a) or untreated (b) with *Pst*I. The diagrams below specify the corresponding lengths (means and standard deviations) expressed in kb. (a) n = 14; (b) n = 3 (n = number of molecules measured). The magnification was about 7500 ×. φX174 single- and double-stranded DNA molecules (5375 bp; Sanger et al., 1977) served as length standards.

*Bam*HI-*Hin*dIII "stop" signals from both ends of the Ω fragment.

(c) In vitro mutagenesis with Ω: general strategy

The use of the Sm^r/Spc^r fragment of pHP45Ω for in vitro mutagenesis is presented in Fig. 5. The plasmid DNA is first linearized and its ends made blunt by the action of DNA polymerase I (Klenow fragment). The mutagenesis can be performed with the purified Ω fragment with *Eco*RI, *Sma*I, or *Bam*HI ends. The use of *Sma*I has been illustrated, since this enzyme generates blunt ends which can be ligated in vitro to any plasmid DNA ends that were, or have been made, blunt. Ω mutants are selected by the Sm^r/Spc^r character, and the position of Ω is determined by digestion with *Bam*HI or *Hin*dIII. Insertion of Ω abolishes endogenous mRNA synthesis, and thus allows the definition and the mapping of transcriptional units. Protein synthesis is also interrupted by the nonsense codons present at the extremities of Ω, allowing a characterization of translational units. The Sm^r/Spc^r fragment can be excised in two manners (Fig. 5). Digestion with *Hin*dIII followed by recircularization leaves behind a 52-bp palindromic sequence containing two *Bam*HI and one *Hin*dIII sites, as well as translational stop codons on both strands in all phases. Alternatively, digestion with *Bam*HI followed by recircularization leaves behind a 10-bp insertion carrying the *Bam*HI site.

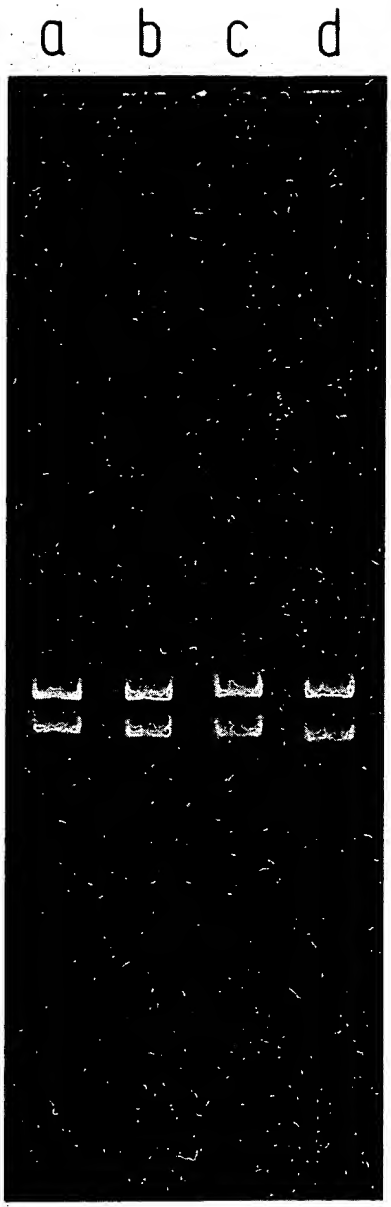


Fig. 4. Restriction analysis of pHP45Ω. The following restriction endonucleases were used to digest pHP45Ω: (a) *Eco*RI; (b) *Sma*I; (c) *Bam*HI; (d) *Hin*dIII. Electrophoresis was carried out on 1% agarose.

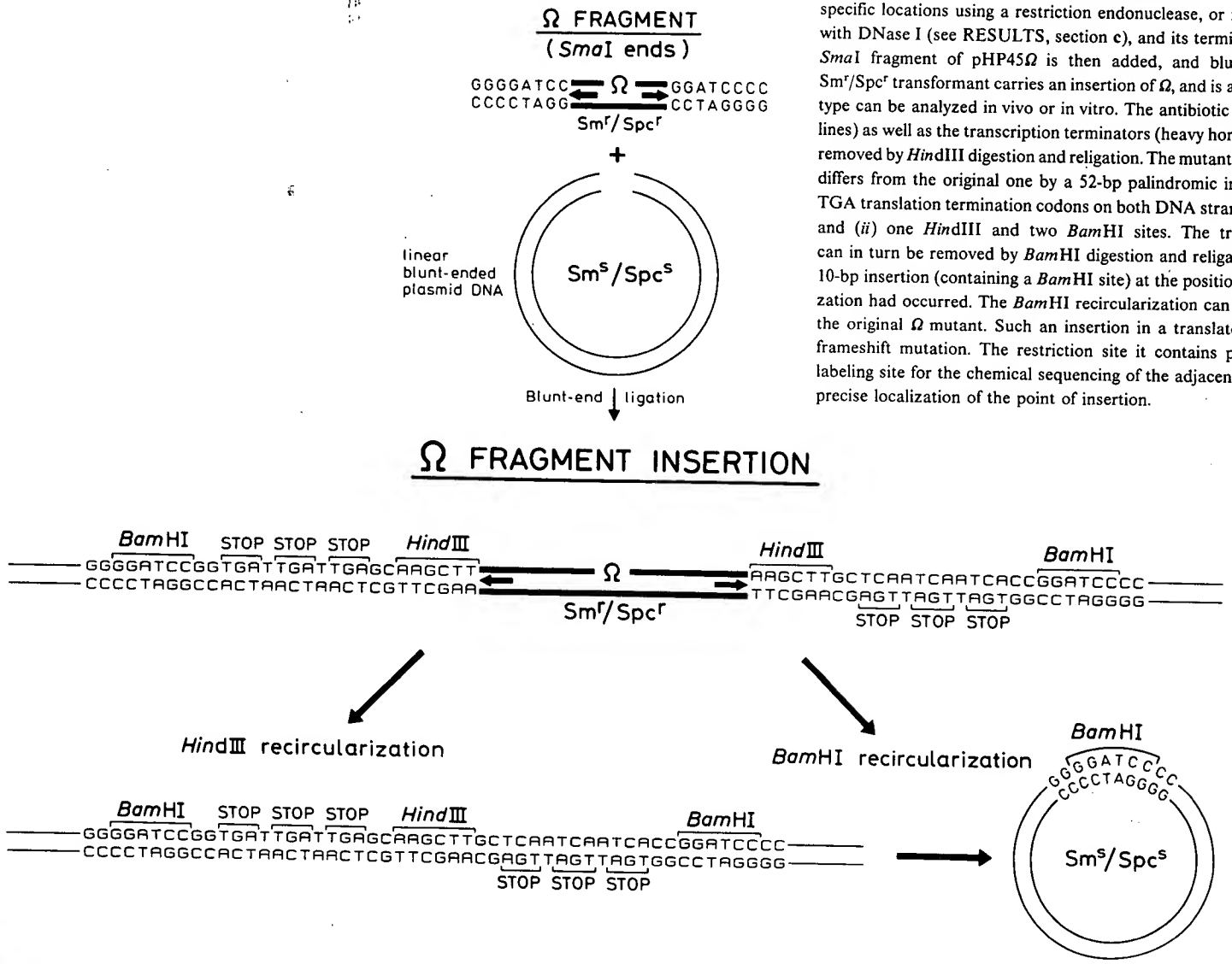


Fig. 5. General strategy for in vitro mutagenesis of circular DNA using the Ω fragment. The plasmid to be mutagenized is linearized enzymatically either at specific locations using a restriction endonuclease, or randomly by digestion with DNase I (see RESULTS, section c), and its termini blunted. The 2.0-kb *Sma*I fragment of pHP45Ω is then added, and blunt-end ligated. Every Sm^r/Spc^r transformant carries an insertion of Ω, and is a mutant whose phenotype can be analyzed in vivo or in vitro. The antibiotic resistance gene (thick lines) as well as the transcription terminators (heavy horizontal arrows) can be removed by *Hin*dIII digestion and religation. The mutant plasmid thus obtained differs from the original one by a 52-bp palindromic insertion containing (*i*) TGA translation termination codons on both DNA strands in all three phases, and (*ii*) one *Hin*dIII and two *Bam*HI sites. The translation terminators can in turn be removed by *Bam*HI digestion and religation, leaving behind a 10-bp insertion (containing a *Bam*HI site) at the position at which the linearization had occurred. The *Bam*HI recircularization can also be performed on the original Ω mutant. Such an insertion in a translated sequence causes a frameshift mutation. The restriction site it contains provides a convenient labeling site for the chemical sequencing of the adjacent DNA and hence the precise localization of the point of insertion.

(d) Translation termination by the Ω fragment

The in vitro insertional mutagenesis with Ω was illustrated using the plasmid p*lac*B235 (Krisch and Allet, 1982) as a target. This plasmid is a derivative of pMC1403 (Casadaban et al., 1980) in which expression of the *lac* operon is under the control of the T4 gene 32 transcriptional and translational regulatory sequences. It thus confers a strong Lac$^+$ phenotype on a host cell deleted for the chromosomal *lac* operon. Plasmid p*lac*B235 carries five recognition sites for the enzyme *Pvu*II, three of which are situated within the structural gene for the gp32-β-galactosidase fusion protein (Fig. 6).

To obtain full size linear plasmid molecules for Ω mutagenesis, p*lac*B235 was digested with *Pvu*II in the presence of EtBr. Purified Ω fragment (*Sma*I ends) was added, and blunt-end ligation was performed. Recipient *E. coli* cells were transformed to Ap^r, Sm^r and Spc^r, and the ability of these cells to ferment lactose was determined on X-gal indicator plates. Some of the Sm^r/Spc^r colonies exhibited a Lac$^-$ character, suggesting that insertion of Ω had abolished expression of the *lacZ* gene. This was confirmed by digesting plasmid DNA of the two classes of transformants (Lac$^+$ or Lac$^-$ with *Bam*HI. All of the plasmids examined carry the Ω fragment at a position previously occupied by a *Pvu*II site (Table I). The Lac$^-$ phenotypes were associated with insertions of Ω at the *Pvu*II sites B and C, within *lacZ* (Fig. 6). The Lac$^+$ insertions mapped at *Pvu*II-D. Among nine Ω mutants examined, no insertions were obtained at the A and E sites, presumably as a result of a less efficient recognition of these sites by *Pvu*II in the presence of EtBr. Insertion of Ω near *Pvu*II-A was, however, achieved by linearizing p*lac*B235 by limited digestion with *Cla*I and filling in the protruding ends with the Klenow enzyme. The plasmids p*lac*B235::ΩZ76 and p*lac*B235::ΩZ78 are Ω insertions into the *Cla*I-B site (at the 279th codon of *lacZ*) in opposite orientations.

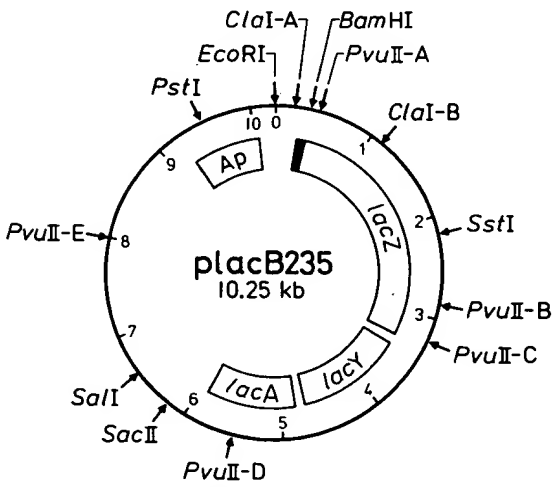


Fig. 6. Map of the plasmid p*lac*B235. In this plasmid (Krisch and Allet, 1982), the bacteriophage T4 gene 32 translational and transcriptional initiation signals (363 bp) are fused in-phase to the *lacZ* gene of pMC1403 (Casadaban et al., 1980). Polypeptide products are indicated by open segments. The first eight codons of *lacZ* are replaced by the first seven codons of gene 32 which are indicated in black at the start of the *lacZ* segment.

The level of β-galactosidase production was determined in vivo for plasmid strains carrying Ω inserted at various sites and with opposite orientations. All Ω mutants within the *lacZ* gene totally abolish its enzymatic activity (Table I).

We have examined the pattern of protein synthesis in cells carrying either p*lac*B235 or its various Ω mutant derivatives. In each case, insertion of Ω within the gp32-β-galactosidase coding sequence results in the synthesis of a polypeptide with an altered electrophoretic mobility (Fig. 7). In every instance the size of the mutant polypeptide is consistent with the introduction of a nonsense codon at the point of Ω insertion (Table I).

(e) Transcription termination by the Ω fragment

(1) RNA-DNA hybridization with Ω mutants of placB235

To obtain a quantitative estimate of the efficiency of transcription termination at the ends of Ω, the rate of transcription of *lacZ* sequences upstream and downstream from the position of Ω was measured by RNA-DNA hybridization, using the plasmids p*lac*B235::ΩZ76 and p*lac*B235::ΩZ78. Exponentially growing MC1061(*Δlac*) cells harboring either of these plasmids were pulse-labeled with [5-^{3}H]-uridine, RNA was extracted and hybridized to DNA immobilized on nitrocellulose filters. RNAs prepared from strains MC1061, MC1061[pMC1403] and MC1061[p*lac*B235] were included as controls. Two restriction fragments, both from p*lac*B235, were purified separately and used as DNA probes:

TABLE I

Properties of *E. coli* strain MC1061 carrying various Ω mutant derivatives of the plasmid p*lac*B235

The isolation and mapping of the various Ω mutants of p*lac*B235 are described in section d of RESULTS. The level of β-galactosidase production was measured according to the method of Miller (1972). β-Galactoside permease (*Lac*Y) activity was assayed on MacConkey-melibiose plates. The sizes of the prematurely terminated gp32-β-galactosidase peptides were determined from the data in Fig. 7.

Plasmid	Site of Ω insertion	Size of gp32-β-galactosidase fusion protein (kDal)	β-Galactosidase activity	MacConkey-melibiose indicator plate reaction
p*lac*B235	none	116	14700; 17400	red
::ΩZ76	*lacZ-Cla*I-B	30 (32)[b]	<1	white
::ΩZ78	*lacZ-Cla*I-B[a]	30	<1	white
::ΩZ2	*lacZ-Pvu*II-B	104 (103)[b]	<1	white
::ΩZ4	*lacZ-Pvu*II-B[a]	104	<1	white
::ΩZ5	*lacZ-Pvu*II-C	115 (115)[b]	<1	white
::ΩZ9	*lacZ-Pvu*II-C[a]	115	<1	white
::ΩZ1	*lacA-Pvu*II-D	116	13600	red

[a] Insertion at the same site but with opposite orientation of Ω.

[b] In parentheses is the expected size of the protein if terminated at point of insertion.

fragment 1 carries 0.82 kb of *lacZ* DNA, between the *Bam*HI and *Cla*I(B) sites (Fig. 6), and acts as an upstream specific DNA probe; fragment 2 contains 1.12 kb of *lacZ* sequences, between *Cla*I(B) and the *Sst*I site. The results of the hybridization experiments are presented in Table II. In strain MC1061[p*lac*B235], 0.18% of the pulse-labeled RNA hybridized to fragment 1, and 0.29% to fragment 2. The higher level of hybridization to fragment 2 is primarily due to its greater size.

In p*lac*B235::ΩZ76 and p*lac*B235::ΩZ78 strains, hybridization to fragment 1 was almost identical to that obtained in the p*lac*B235 strain, demonstrating that Ω had no influence on the rate of transcription of upstream sequences. In both cases, however, hybridization to fragment 2 underwent a dramatic reduction, representing only about 0.01% of the input radioactivity. Hence, the presence of Ω reduces the transcription of downstream *lacZ* sequences down to a value equivalent to only 4–6%

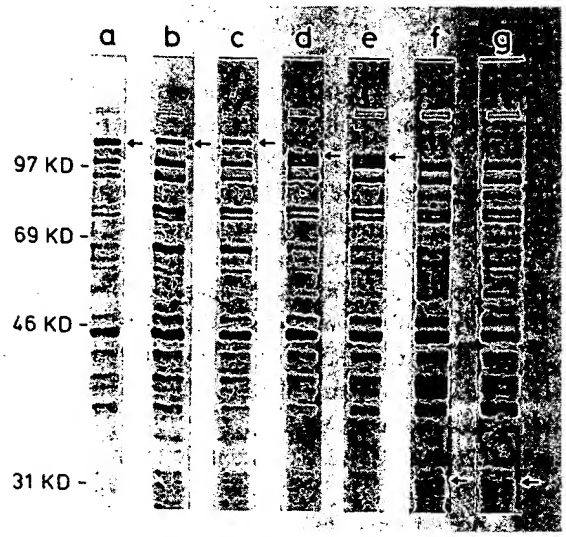
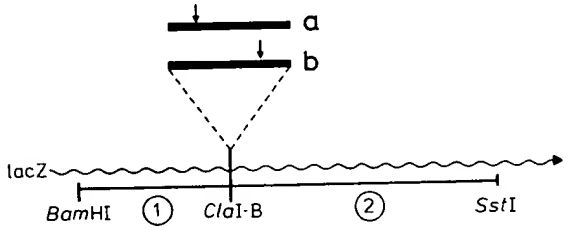


Fig. 7. Ω-mediated translation termination. The bacterial cultures were grown at 37°C in M9 medium supplemented with ampicillin (20 μg/ml) and casamino acids (0.2%). While still in exponential phase, the bacteria were harvested by centrifugation, and the cellular proteins were labeled with a mixture of [^{14}C]amino acids as described by Krisch and Selzer (1981). The lysates were analyzed by PAGE. A Coomassie-brilliant-blue-stained 10% SDS-polyacrylamide gel with the protein bands of MC1061 strains carrying Ω mutant derivatives of p*lac*B235 is shown. Comparable results were obtained upon autoradiography of the gel. The plasmids carried by the strains were p*lac*B235 (lane a); p*lac*B235::ΩZ2 (lane b); p*lac*B235::ΩZ4 (lane c); p*lac*B235::ΩZ5 (lane d); p*lac*B235::ΩZ9 (lane e); p*lac*B235::ΩZ76 (lane f); p*lac*B235::ΩZ78 (lane g). The gp32-β-galactosidase fusion peptides are designated by arrows. Their M_rs were estimated using phosphorylase B (97 kDal), bovine serum albumin (69 kDal), ovalbumin (46 kDal) and carbonic anhydrase (31 kDal) as standards.

TABLE II

Effect of the Ω insertion on synthesis of *lacZ* mRNA

The diagram represents a section of the *lacZ* gene in the p*lac*B235 plasmid, between the unique *Bam*HI and *Sst*I sites (see Fig. 6). The insertion of Ω (thick line) into the filled-in *Cla*I-B site of p*lac*B235 to form the p*lac*B235::ΩZ76 and p*lac*B235::ΩZ78 plasmids is indicated by letters a and b, respectively. The direction of transcription of *lacZ* is represented by a wavy arrow and the probe fragments 1 and 2 are indicated with encircled numbers.



Plasmid	Fragment 1		Fragment 2		Ratios	
	cpm	% input (× 100)	cpm	% input (× 100)	2/1	2/1 (norm)
(1)	(2)	(3)	(4)	(5)	(6)	(7)
none	16	—	17	—		
pMC1403	32	0.5	35	0.6		
p*lac*B235	1085	17.7	1784	29.4	1.66	1.00
::ΩZ76	644	15.2	61	1.0	0.07	0.04
::ΩZ78	728	15.7	97	1.6	0.10	0.06

The average of [^{3}H]RNA counts hybridized to duplicate filters with fragments 1 or 2 DNA are given for each pulse-labeled strain in columns 2 and 4. Hybridization of [^{3}H]RNA from MC1061 to fragment 1 or 2 DNA was at the level of background (^{3}H cpm's on blank filters). For each set of filters, the hybridized radioactivity is also expressed as a proportion of total input (columns 3 and 5). The inputs were 0.678×10^6, (MC1061); 0.879×10^6, (MC1061[pMC1403]); 1.241×10^6, (MC1061[p*lac*B235]); 0.820×10^6, (MC1061[p*lac*B235::ΩZ76]); and 0.897×10^6, (MC1061[p*lac*B235::ΩZ78]). The ratios of the levels of hybridization to the two fragments (column 6) were normalized to the value observed with p*lac*B235, as to take into account the difference in the efficiency of hybridization to the two probes (column 7).

of the original mRNA synthesis (Table II, last column).

(2) Polarity of Ω in the lac *operon*

In the plasmid p*lac*B235, transcription initiation prior to *lacZ* results in a polycistronic mRNA which contains this gene as well as *lacY* and *lacA*. Transcription termination at an Ω fragment inserted within *lacZ* should result in a polar effect on the expression of *lacY* and *lacA*. The expression of *lacY* can be easily assayed by the ability of cells to utilize melibiose as a carbon source (Miller, 1972). This α-galactoside requires the LacY protein for efficient transport into cells grown at 37°C. The various Ω mutants of p*lac*B235 were thus tested on MacConkey-melibiose indicator plates (Table I). The host strain carrying the plasmid p*lac*B235 gave red colonies as expected. All Ω mutants in *lacZ* gave white colonies, indicating that they were unable to express *lacY*. An insertion of Ω beyond *lacY* (p*lac*B235::ΩZ1) had no effect on *lacY* gene expression. These genetic results confirm that transcription termination is efficiently mediated by the Ω fragment.

DISCUSSION

The most commonly used approach to inactivate genes cloned in *E. coli* is transposition mutagenesis. Insertion mutants of this type are easy to detect, usually because of a selectable marker, such as an antibiotic resistance gene, present in most transposons. They can be mapped by making use of the restriction sites introduced by the transposable element. Several difficulties, however, are associated with the use of transposons as mutagens. First, some transposable elements exhibit a bias for the position of integration into the target molecule, either in a sequence-specific manner (Tn*10*: Halling and Kleckner, 1982; IS*4*: Klaer et al., 1981; Tn*7*: Lichtenstein and Brenner, 1982), or through a strong preference for A/T rich regions (Meyer et al., 1980; Miller et al., 1980). Second, transcriptional activity into adjacent DNA has been reported (Heffron et al., 1979; Simons et al., 1983; Churchward, G., pers. commun.), sometimes complicating the phenotypic and genetic characterization of insertion mutants. Finally, once inserted into the target molecule, transposable elements have the capacity to generate DNA rearrangements such as deletions or inversions.

An alternative, in vitro procedure has been developed by Heffron et al. (1978). It makes use of

short synthetic oligodeoxynucleotides (*Eco*RI "linkers") as mutagenic modules, which are inserted at double-strand cleavages randomly generated with DNase I. Thus, the event that generates a mutation in a cloned gene simultaneously allows physical mapping by a simple restriction enzyme digestion. This approach, however, involves numerous biochemical steps to enrich and to screen for linker-mutagenized molecules.

The construction of a DNA fragment that facilitates in vitro insertional mutagenesis has been described in this communication. This fragment enables one to mutagenize under conditions where use of transposons is not appropriate (when mutations are to be introduced at specific sites), or when simple restriction linkers are not sufficient. The most important aspect of Ω mutagenesis is the selectable introduction of translational and transcriptional stop signals. This allows the definition of both translational and transcriptional units within cloned DNA. Ω can also be used when inactivation of RNA or protein synthesis downstream from a given location, such as a restriction site, is desired.

In its simplest form (insertion followed by excision) Ω mutagenesis allows the selectable transposition of DNA linker sequences into recombinant DNA molecules (Fig. 5). This makes in vitro mutagenesis with DNA linkers accessible to simple bacterial genetics and cloning techniques. The presence of the antibiotic-resistance gene between the DNA linker sequences implies that any Sm^rSpc^r colony contains a mutagenized plasmid. It allows rare events to be detected, as was seen in the generation of plasmids p*lac*B235::ΩZ76 and p*lac*B235::ΩZ78, in which less than 5% of the plasmid population had been cut with *Cla*I.

By analogy with Ω, selectable DNA fragments carrying other regulatory sequences could be assembled to mutagenize, and to modulate the level of expression of cloned DNA sequences. We have recently constructed a fragment in which the Sm^r/Spc^r gene is flanked by inverted repeats with prokaryotic promoters (H.M.K. and P.P., unpublished). A similar fragment, with a selectable gene expressed both in prokaryotic and eukaryotic cells flanked by eukaryotic promoters, would also be potentially very useful.

The Ω fragment has a structure reminiscent of that of many bacterial transposons: an antibiotic resistance gene flanked by an inverted repeat sequence. This structure can be transposed in vitro into recombinant DNA molecules. We propose to call it an "interposon".

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